Power RNA Analysis

Nuclease Protection Assays Offer Sensitivity and Versatility

By Ellen Prediger, Dir. Scientific Communication and Technical Services

- **Simultaneously assay a single RNA sample with multiple probes, now including oligonucleotides**
- **Single tube reaction with no phenol extraction or proteinase K digestion**
- **Detect as little as 5 femtograms of target mRNA**
- **Use up to 100 µg of sample RNA – poly(A) selection is usually unnecessary**
- **Map mRNA termini and intron/exon junctions**
- **Distinguish between members of multigene families**
- **Tolerant of partially degraded RNA**
- **Any of Ambion's NPA kits may be used with radiolabeled or nonisotopically labeled probes**

Nuclease protection assays (NPAs), including both ribonuclease protection assays (RPAs) and S1 nuclease assays, are an extremely sensitive method for detection, quantitation and mapping of specific RNAs in a complex mixture of total cellular RNA. The reaction can be 10 to 100+ fold more sensitive than Northern analysis, and is easier to optimize for quantitation and multiprobe analysis than RT-PCR. Ambion's new Multi-NPA™ Kit further extends the advantages of NPAs by allowing use of oligonucleotide probes alone or in conjunction with RNA probes to extend the size range of multiple probes and to probe for transcripts when very little sequence information is available.

How the assay works

The basis of NPAs is solution hybridization of a single-stranded, discrete sized, antisense probe(s) (radiolabeled or nonisotopically labeled) to a complex mixture of RNA. The small volume solution hybridization is far more efficient than membrane-based hybridization and can accommodate up to 100 µg of total or poly(A) RNA. After hybridization, any remaining

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Ambion® TechNotes™ from The RNA Company™

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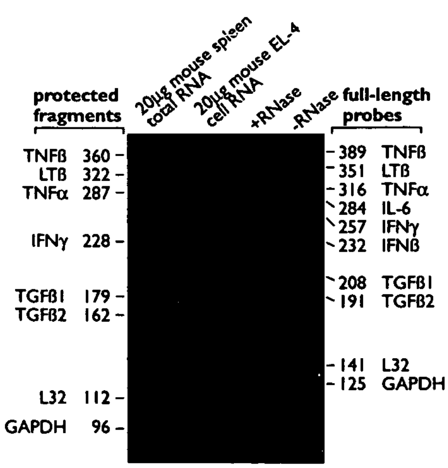

Figure 1. RPAs were performed using Ambion's RPA II™ Kit and ^{32}P-labeled RNA probes generated from RiboQuant™ Multiprobe Template Set, mCK-3(PharMingen) using Ambion's MAXIscript™ Kit. The reactions were assessed on a 15 cm, 5% polyacrylamide/8M urea gel that was subsequently vacuum dried and exposed to film for 5 days at room temperature. (RiboQuant is a trademark of PharMingen.)

Construct Transcription Templates Without Cloning

by Tiffany J. Smith, M.S.
Product Applications Specialist

Synthesis of an RNA probe by *in vitro* transcription requires a double-stranded DNA template containing the specific cDNA of interest adjacent to a promoter sequence. Many commercially available plasmid vectors incorporate phage RNA promoter sequences on each sides of the multiple cloning region. Such plasmids carrying a cDNA insert can easily serve as transcription templates. However one must first insert the cDNA of interest into the plasmid, select, grow, and purify it. Once purified, the plasmid must be linearized with an appropriate restriction enzyme and repurified.

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New! GlycoBlue™ Coprecipitant

new PRODUCT!

- **Blue color increases visibility of sample pellet**
- **Increases pellet mass**
- **Quantitative recovery of any small amount of nucleic acid**
- **Prevents pellet loss in nuclease protection assays**

GlycoBlue consists of a blue dye covalently linked to a branched chain carbohydrate, glycogen. Glycogen will precipitate RNA and DNA when used at a final concentration of 10-50 µg/ml in the presence of 0.5M ammonium acetate and isopropanol or ethanol. GlycoBlue can be used as a coprecipitant in nuclease protection assays at 1/100 volume. Glycogen offers a means of enhancing precipitation without adding appreciable amounts of exogenous nucleic acids to the sample. For this reason, it is preferable to yeast RNA as a coprecipitant for applications where nucleic acid mass is being assessed or added nucleic acid could interfere or compete with subsequent enzymatic reactions. While the glycogen adds mass to the nucleic acid pellet, the blue dye makes the pellet more visible. These features make GlycoBlue an indispensable reagent in nuclease protection assays to limit pellet loss.

Ambion's glycogen, isolated from a biological source, is treated with Proteinase K and SDS to remove any contaminating nucleases, then phenol:chloroform extracted, ethanol precipitated, resuspended in nuclease-free water and quantified by an enzymatic assay. The glycogen is guaranteed RNase-, DNase- and proteinase-free. GlycoBlue is compatible with most nuclease protection assay procedures and is included in Ambion's RPA II™ and Multi-NPA™ Kits. ■

Ordering Information	cat#	size	price
GlycoBlue™ Coprecipitant (15mg/ml)	9515	1 x 300 µl	$35.00
	9516	5 x 300 µl	$95.00

Preparing DNA Template for RNA Probe Synthesis

One of the major reasons for failed transcription reactions is the presence of inhibitors in the DNA template. Inhibitors include RNases from restriction enzymes and trace amounts of EtOH or salts. The following instructions for preparing DNA template should limit the presence of inhibitors. Note that PCR templates typically do not contain contaminants that will compromise transcription and can usually be used directly without further purification.

1. Plasmid transcription templates should be digested with the appropriate restriction enzyme to linearize the plasmid near the end of the insert to be transcribed.
2. Treat the digest with 100 - 200 µg/ml proteinase K and 0.5% SDS at 50°C for 30 - 60 minutes.
3. Remove degraded proteins by extracting the prep with an equal volume of phenol:chloroform or phenol:chloroform:IAA. The initial aqueous volume can be increased to create volumes that are easier to work with.(See "Tips from the Bench," page 17)
4. Precipitate with 1/10 volume 5M NH_4OAc and 2.5 volumes EtOH.
5. Wash pellet with 70% EtOH. (See "Tips from the Bench," page 17)

Alternatively, any digestion can be cleaned up with Ambion's GeniePrep™ Plasmid DNA Purification Kit (Catalog #12100). ■

Increasing RNA Probe Sensitivity

The success of RNA analysis often depends on the sensitivity of the probe used. That is, the overall sensitivity of any assay can be increased by increasing the specific activity (label/µg) of the probe.

One of the easiest ways to increase probe sensitivity is to increase probe length, thus allowing more labeled nucleotides to be incorporated. Ambion recommends probe length to be between 100 and 800 bases for nuclease protection assays, but some researchers have used probes as large as 1000 bases. In Northern analysis or any blot hybridization, probe length is not an issue.

For radiolabeled RNA probe synthesis, Ambion recommends using ^{32}P-UTP or ^{32}P-CTP with a specific activity of 800 Ci/mmol, 10-20 mCi/ml. To maximize probe specific activity, little to no corresponding unlabeled nucleotide should be added to the transcription reaction. Many believe that using a higher specific activity ^{32}P label such as 3000 Ci/mmol, will significantly increase the specific activity of the probe. While this is theoretically true, it can be impractical. Isotopes with such high specific activities are sold significantly more dilute (3.3 µM vs. 12.5 for an 800 Ci/mmol, 10 mCi/ml). One would have to add an entire stock vial to a single reaction in order to achieve the minimum amount of limiting (labeled) NTP (>3 µM) to allow for full-length transcription.

Ambion's new CU minus™ technology helps to overcome this dilemma. By providing vectors and promoter conversion primers that omit C or U nucleotides in the first 10 bases of the nascent transcript where synthesis most frequently aborts, lower concentrations (as little as 0.3 µM) of higher specific activity labeled NTPs (3000 or 6000 Ci/mmol) can be used. The use of this technology can increase probe sensitivity 7.5 times over the standard RNA probe (see related article in TechNotes 4:2 at www.ambion.com).

More is not always better

It should be noted that in nuclease protection assays, simply adding more probe does not increase the sensitivity of the assay. There should be enough probe to be in molar excess of the target message. Any additional probe will not hybridize and will only add to the background.